AN ELECTRON MICROSCOPIC STUDY OF THE CHORIOALLANTOIC MEMBRANE FOLLOWING INFECTION WITH ROUS SARCOMA VIRUS

P. R. SWEENY and R. BATHER

From the Department of Biology, University of Saskatchewan, Saskatoon, Canada. Dr. Bather's present address is Saskatchewan Research Unit of the National Cancer Institute, University of Saskatchewan, Saskatoon, Canada

ABSTRACT

Infection of the chick chorioallantoic membrane (CAM) with Rous sarcoma virus (RSV) has been thought by earlier workers (12, 20) to result in the transformation of the ectoderm and then the mesoderm of that organ. In the present study, CAM were infected with 10^4 PFU (pock-forming units) of RSV (Bryan high titre strain) and collected for electron microscopy at 2, 4, and 6 days postinfection. Observations of the fine structural changes in the CAM after RSV infection support a singular role of the mesenchyme in the initiation of the tumors. The ectodermal hyperplasia often associated with RSV tumors of the CAM appears to be a secondary response to the alteration of the underlying mesenchyme. These findings are discussed in detail, and an alternate course of RSV transformation of the CAM by way of the vascular bed is suggested.

The chorioallantoic membrane (CAM) was first used by investigators interested in Rous sarcoma virus (RSV), and the latter's oncogenic capacities were described in 1911 by Rous (25). In the ensuing years many investigators have used the CAM for a variety of studies involving RSV. A few papers have dealt with the early microscopic changes in this organ after RSV infection, the most detailed studies being those of Keogh (12) and Prince (20). Many descriptive confirmations have appeared in the literature subsequently (3, 6, 26, 29, 30). Most investigators believe that the initial action of the virus is on the ectodermal cells which causes their proliferation with concomitant viral replication, and that the release of this virus subsequently infects the underlying mesenchyme and affects its proliferation. These two attendant events are thought to produce the characteristic "pock" tumors on the CAM.

There is not available in the literature, however, any detailed study of the early fine structural changes in the CAM following RSV infection. An investigation along these lines was, therefore, undertaken and is reported in this paper. The information to be presented suggests that the ectoderm may not be involved as was thought by earlier workers but that the primary action of the virus may be on the mesodermal derivatives. An alternative route of viral infection of the CAM is suggested on the basis of the findings presented.

MATERIALS AND METHODS

Virus was obtained from Rous tumors grown in the leg muscle of 2–3-wk-old chicks by weekly transfer of tumor homogenate. The chicks were obtained through the courtesy of Dr. C. leQ. Darcel, from his flock of East Lansing Line 15 White Leghorns maintained in isolation at the Canada Department of Agriculture, Animal Diseases Research Institute (Western), Lethbridge, Alberta.

All eggs used in these studies were obtained from a flock of white leghorns maintained in isolation by the Poultry Science Department, University of Saskatchewan. These eggs were used for routine RSV assays and had a uniform response to the virus; very few of them showed resistance. The chorioallantoic membranes were dopped by the creation of an artificial air space after 9 days of incubation and inoculated with 0.1 ml of crude RSV (Bryan high titre strain). The virus was prepared by homogenizing tumor tissue in 10 volumes of Hanks' balanced salt solution (BSS) and treated with hyaluronidase (0.1 mg/ml) for 15–30 min at 37°C for reduction of viscosity. Cells and debris were deposited in the centrifuge with two cycles of 1500 *g* for 10 min each. The supernatant was used to inoculate the eggs and was so diluted that approximately 10^4 PFU (pock-forming units) were placed on the CAM. Control eggs were inoculated with 0.1 ml BSS.

After infection, the membranes were collected at 2, 4, and 6 days and fixed in phosphate-buffered osmium tetroxide (1%) with 0.54% glucose (15). After fixation the collected membranes were examined under a dissecting microscope, and care was taken to select areas showing early lesions, large discrete pocks, and normal-appearing areas. All tissues were dehydrated and embedded in Epon (14) and were then appropriately oriented for sectioning.

1 μ sections were collected from all blocks after thin sectioning, stained with methylene blue azure II (21), and viewed with the light microscope. Thin sections obtained with the Porter-Blum ultramicrotome I were mounted (unsupported) on 200-mesh copper grids, stained with lead acetate and uranyl acetate (5.0% in methanol) singly or as a double stain, and viewed in a Phillips electron microscope, model 100B.

OBSERVATIONS

The structure of the CAM as seen in the light microscope is shown in Fig. 1. It must be emphasized that the thickness of this organ varies over vast ranges (150 μ–1 mm) and that the differences in thickness are predominantly due to variations in the mesodermal cell population and its associated vascular bed. The fine structural characteristics of this organ have been well documented by Leeson and Leeson (13), and our findings of normal CAM 2, 4, and 6 days after dropping generally confirm their observations on the membrane at 9–15 days of development. It was noted

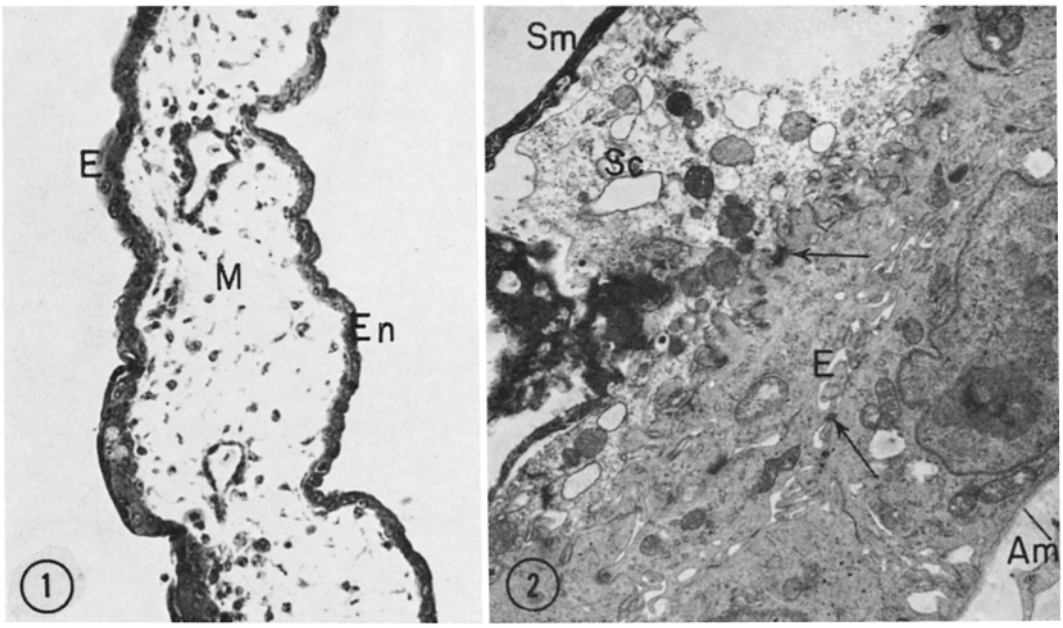


FIGURE 1 Light micrograph of control CAM, 2 days postdropping. Note ectoderm (*E*), mesoderm (*M*), and endoderm (*En*). Hematoxylin and Eosin. × 400.

FIGURE 2 Electron micrograph of normal CAM, 2 days postdropping. Note shell membrane (*Sm*), degenerate sinusoidal cells (*Sc*), two-layered ectoderm (*E*), and adepidermal membrane (*Am*). Desmosomes are evident at cell boundaries (arrows). OsO_4. × 5500.

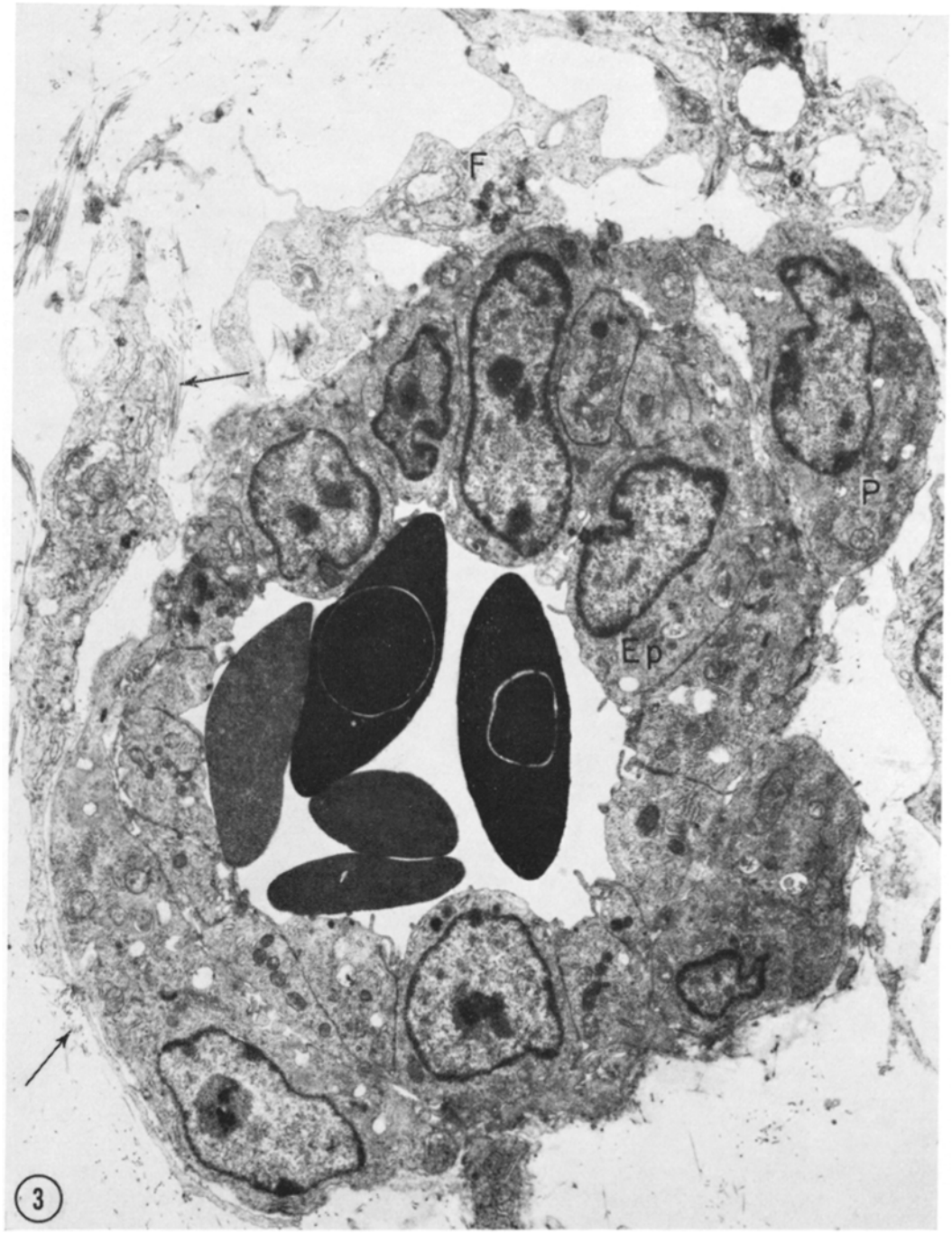


FIGURE 3 Low-power electron micrograph of small CAM vessel showing cuboidal-like endothelium (*Ep*), pericytes (*P*), and closely associated fibroblasts (*F*). Small amounts of collagen are visible (arrows). OsO_4 . × 6000.

however, that following dropping of this membrane, with or without infection, the cells of the sinusoidal endothelium underlying the eggshell membrane seem to undergo degenerative changes, the ectodermal cells themselves appearing normal (Fig. 2). The latter are disposed in two distinct layers as flattened cells, and scattered desmosomal connections exist between these cells and the overlying sinusoidal cells. Between the ectodermal cells there are distinct intercellular spaces with interdigitating microvilli along all cell borders. The double epithelial sheet of ectoderm is separated from the underlying mesoderm by a distinct and continuous adepidermal membrane (Fig. 2).

The mesoderm is characterized by widely scattered fibroblasts with occasional erythrocytes and leukocytes. Coursing through this cell population is the vascular bed of the CAM and, in the areas studied, the vessels vary from 10 μ to 1 mm or more in diameter. No smooth muscle was observed in the walls of the smaller vessels at any time. These smaller vessels penetrate the ectoderm, their lumens being continuous with the sinusoidal spaces between the ectoderm and shell membrane as described by Leeson and Leeson (13). The endothelium of the smaller vessels is more cuboidal than squamous in appearance. Peripheral to the endothelium is a population of flattened cells with occasional cells that may perhaps represent "pericytes" (19) (Fig. 3). The larger vessels show very complex cell relationships, i.e. definitive endothelium with external smooth muscle, fibroblasts, and a preponderance of intercellular collagenous fibrils.

The intercellular area of the mesoderm of the control CAM at the times studies appeared to contain relatively few collagen fibrils.

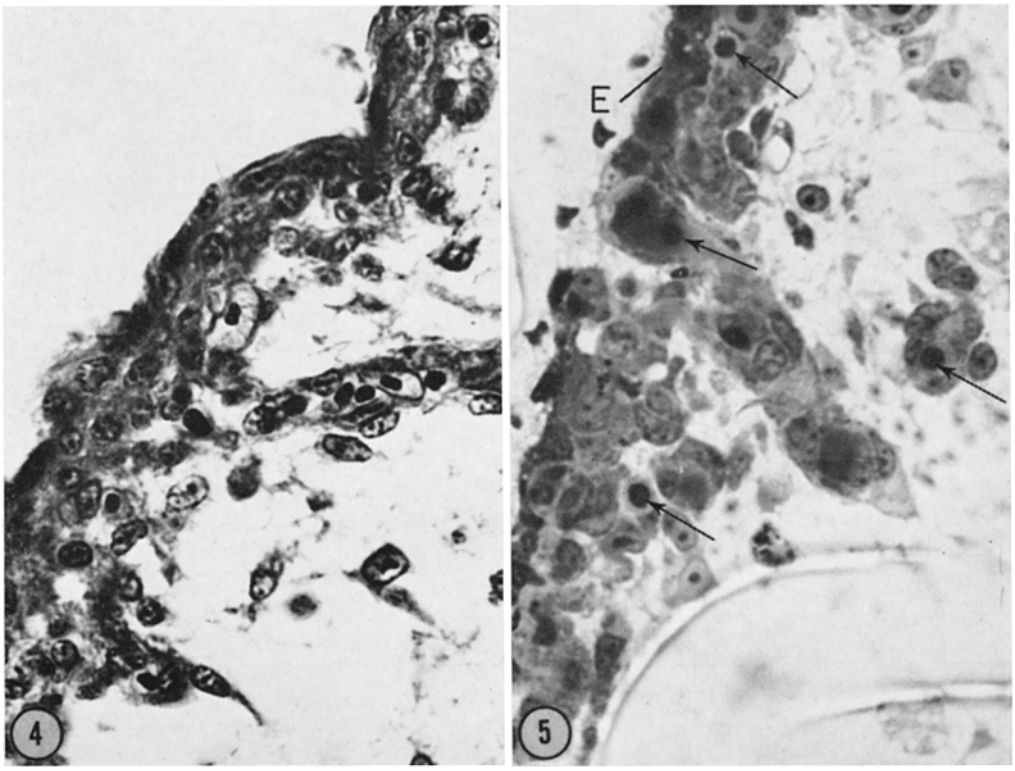


FIGURE 4 Light micrograph of CAM ectoderm, 2 days postinfection, showing apparent thickening to four to six cell layers. Hematoxylin and Eosin. × 1500.

FIGURE 5 Light micrograph of CAM ectoderm. Note two distinct cell layers: an outer layer of dense ectoderm (*E*) underlain by a layer of lighter cells with enclosed erythrocytes (arrows). Epon-embedded and stained with methylene blue azure II. × 1500.

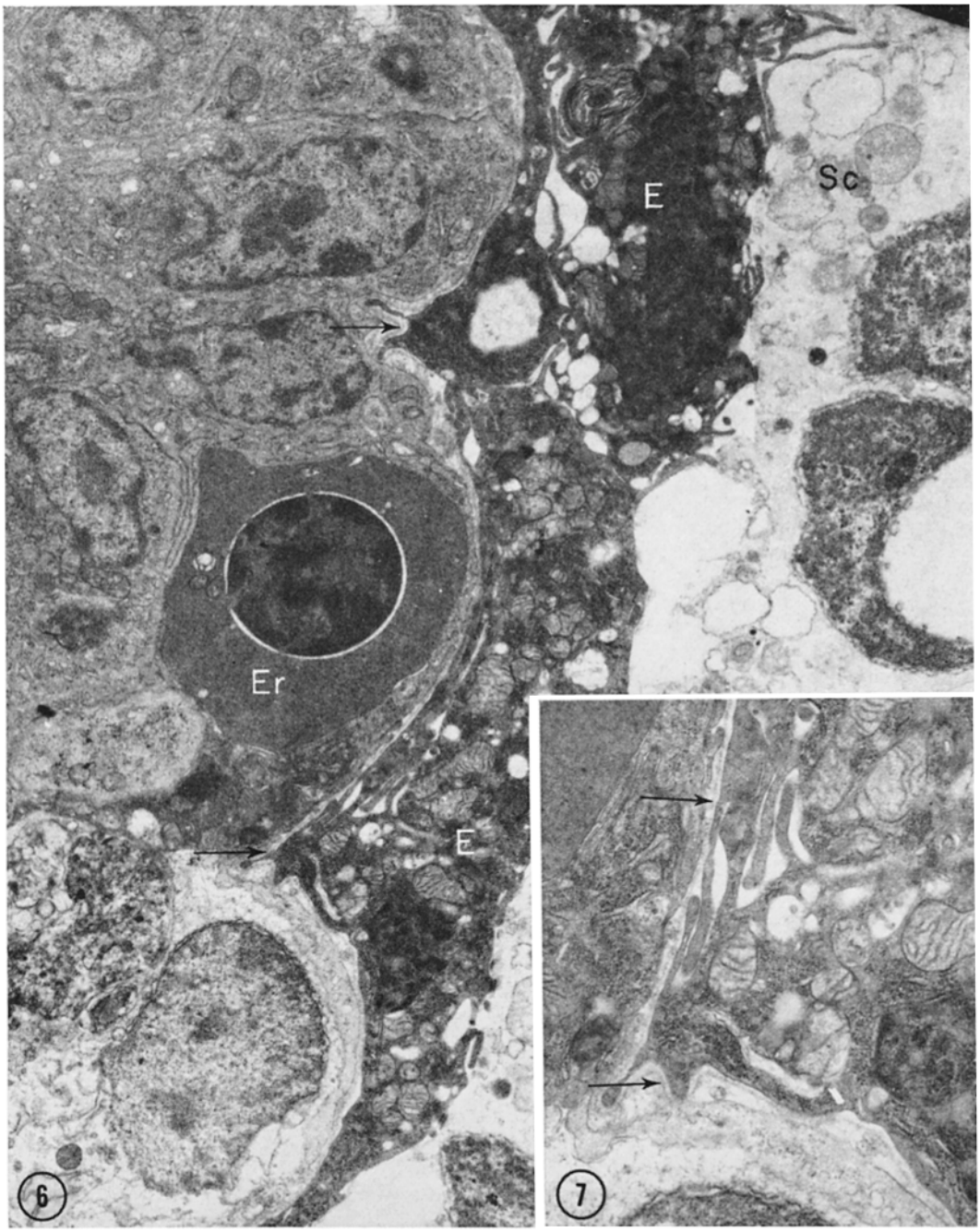
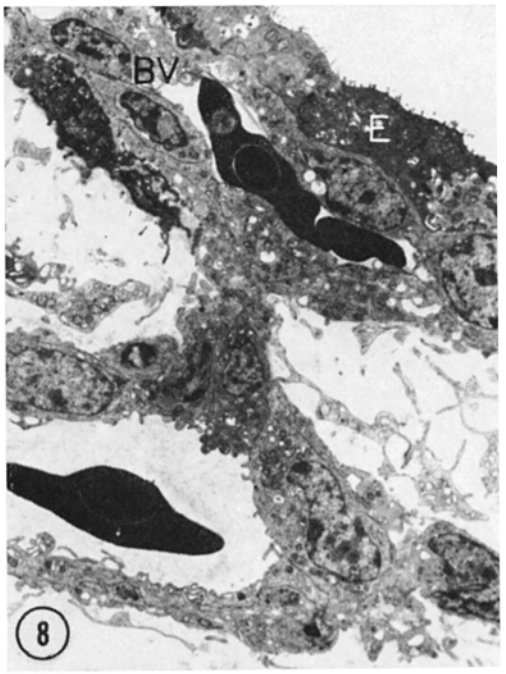


FIGURE 6 Electron micrograph of CAM, 2 days postinfection, showing electron-opaque ectoderm (*E*) and degenerate sinusoidal cells (*Sc*). Underlying these is an electron-transparent cell population with an enclosed erythrocyte (*Er*), and these cells are separated from the overlying ectoderm by the adepidermal membrane (arrows). OsO_4. × 9000.

FIGURE 7 Enlargement of junction zone in Fig. 6 to show adepidermal membrane (arrows). OsO_4. × 13,000.



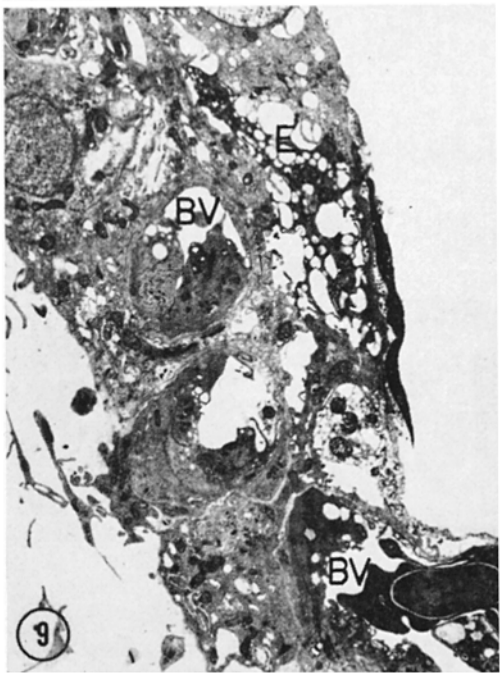


FIGURE 8 Low-power electron micrograph of CAM, 4 days postinfection, showing penetration of blood vessel (*BV*) into the ectoderm (*E*). OsO_4. × 2500.

FIGURE 9 Low-power electron micrograph of CAM, 4 days postinfection, showing blood vessels (*BV*) within ectoderm (*E*). OsO_4. × 3000.

INFECTED MEMBRANES: Histologically the only observable change in the CAM at 2 days postinfection is an apparent thickening of the ectodermal epithelium to three or four cell layers (Fig. 4). However, a different picture was observed when a sample from this same CAM, which had been Epon-embedded, was sectioned at 1 μ and stained with methylene blue azure II. It became apparent that the ectoderm has two distinct cell layers: an outer, densely staining layer one or two cells thick ,and an underlying, more lightly stained layer of variable thickness with enclosed erythrocytes (Fig. 5).

Electron microscopic examination of these infected chorioallantoic membranes revealed the sinusoidal cells to be highly vesiculated with degenerative characteristics and underlain by the definitive ectodermal cells (Fig. 6). Below the ectoderm and separated from it by the adepidermal membrane is a third population of cells resembling vascular epithelium and containing erythrocytes (Figs. 6 and 7). This observation is not an occasional one, because this subepidermal cell aggregation was seen in varying degrees on all sections of CAM 2 days postinfection. In some cases it perhaps represents an early phase (initiation center) of pock formation. Control CAM exposed to BSS showed no such subepidermal cell population.

Occasional virus particles can be seen at this time in the extracellular compartment of the mesodermal area of infected CAM, but no virus was ever seen in the ectodermal areas at 48 hr postinfection.

At 4 days postinfection, centers of pock formation are visible (following osmium tetroxide fixation) as dark spots on the CAM. This permits specific selection of areas and thus affords one the possibility of selecting well-established lesions as well as what appear to be small initiation centers.

At 4 days postinfection the tissues show the extent to which mesodermal derivatives have penetrated into the ectoderm proper (Figs. 8 and 9). In some cases the vessels were seen to lead directly into the ectoderm and to show lateral branching (Fig. 8). In others these lateral intraectodermal vessels were seen in cross-section (Fig. 9); this implies a very tortuous course through the ectoderm. Other cells can be seen subectodermally, but whether these are vascular is uncertain although they do appear in some cases to be surrounded by a basement lamina and their cyto-

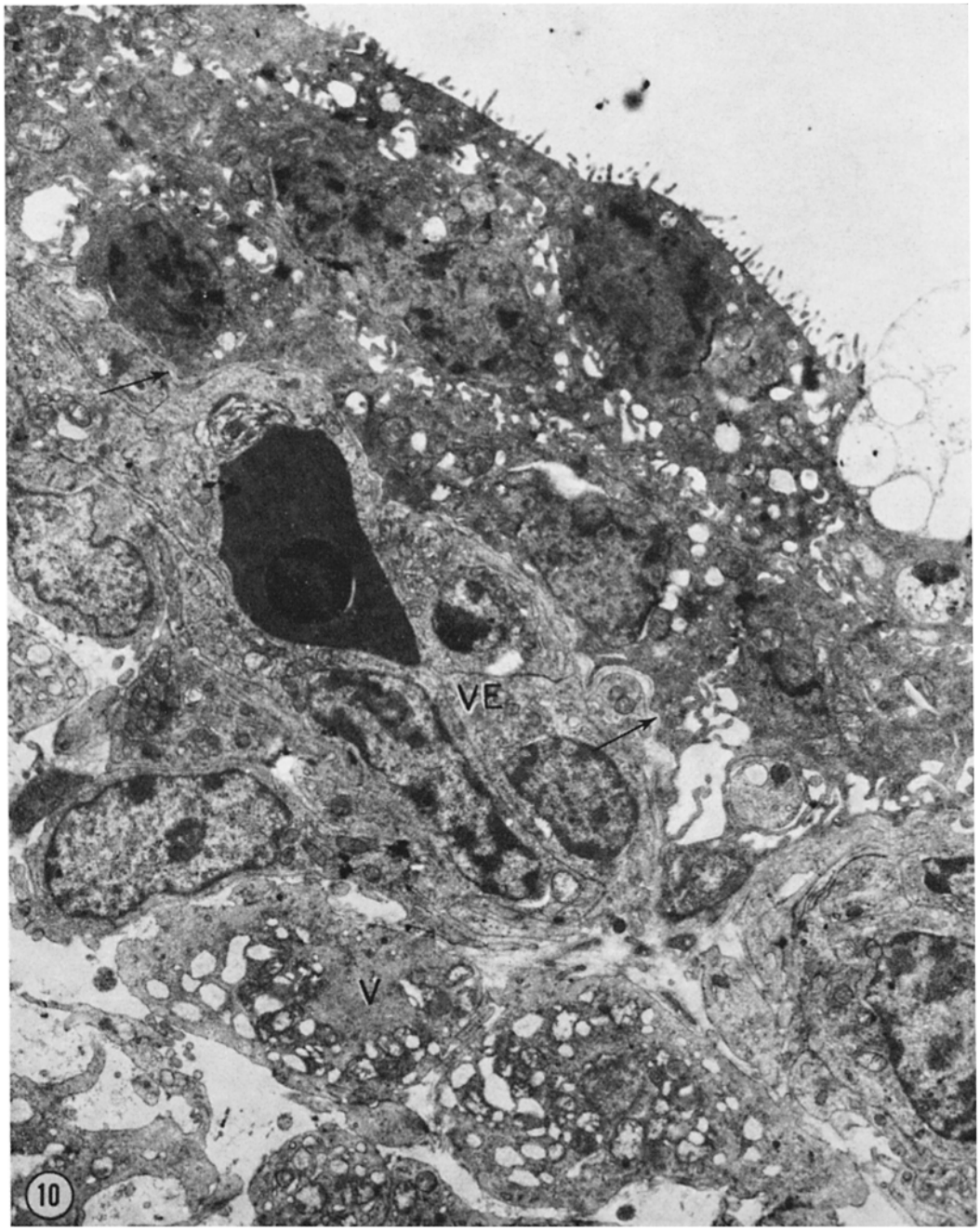


FIGURE 10 Electron micrograph of a discrete pock, 4 days postinfection. The ectoderm can be seen to be separated by the adepidermal membrane (arrows) from the underlying cell mass. The latter population shows two major cell types: vascular endothelial cell (*VE*) and a highly vesiculated cell (*V*). OsO_4. × 5000.

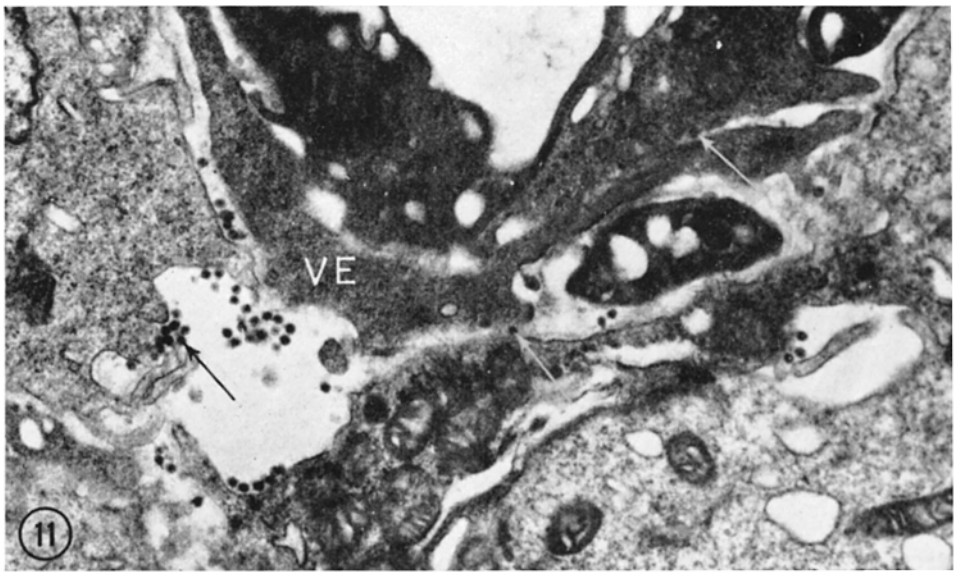


FIGURE 11 Electron micrograph showing virus in ectoderm of CAM, 4 days postinfection. The virus is located close to the vascular endothelium (*VE*) (white arrows) and in the ectodermal cell area (black arrow). OsO_4. × 17,000.

plasmic characteristics closely resemble those of vascular cells. These features are found in regions of small initiation centers and have not been observed in the control preparations.

Collected specimens resembling discrete pocks are more revealing in their cytological characteristics (Fig. 10). In these discrete pocks one can readily identify three distinct cell types. The ectoderm with the denser ectodermal cells is two or sometimes three layers thick and is directly underlain by a compact mass of vascular endothelium often enclosing red blood cells. This latter cell population is separated from the overlying ectoderm by a distinct adepidermal membrane. Deeper to this cell population is a highly vesiculated cell type with extensive cytoplasmic projections and irregular mitochondria. Virus is also discernible at 4 days in most sections but is usually in mesodermal areas. When virus was seen within the ectodermal cell population, it was always extracellular and was sometimes closely associated with the vasculature within the ectoderm (Fig. 11).

At 6 days postinfection true pocks which are readily identifiable show an aggregate of all cell types similar to those described, and some pocks possess a true ectodermal thickening. Within the ectoderm of small pocks, however, cells which may be of vascular origin are still discernible (Fig. 12), but all distinct relationships within these areas are lost. Within the subectodermal area, however, three distinct cell types are visible. The cells of the vasculature are identifiable by their anatomical position. However, the true external boundaries of the vessel itself are not so clearly defined, and in the perivascular area within a pock distinct cell types are visible (Fig. 13). Some cells show a relatively dense cytoplasm, rough endoplasmic reticulum, and other characteristics of normal fibroblasts. Other cells that are quite distinct from the former show excessive vesiculation of the endoplasmic reticulum and some small vesicles as well as cytoplasmic ribosomes are present. Some of these cells contain electron-opaque materials within the dilated endoplasmic reticulum. In addition to these characteristic cells, large amounts of intercellular collagen have become visible.

DISCUSSION

Investigations by many workers have implicated the ectoderm in the initial phase of RSV infection of the CAM (3, 6, 12, 20, 26, 29, 30). Our electron microscopic studies do not confirm these findings; in fact, they indicate that the cells of the mesoderm

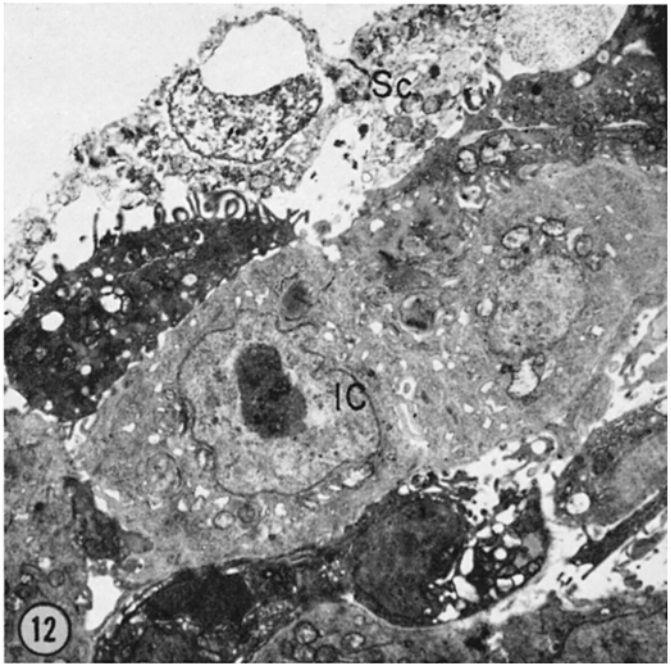


FIGURE 12 Low-power electron micrograph of CAM ectoderm, 6 days postinfection, showing degenerate sinusoidal cells (*Sc*), electron-opaque ectoderm proper, and lightly stained infiltrating cells (*IC*). Compare to cells in Fig. 6. OsO_4. × 4500.

may be primarily involved in the production of the lesions within this organ. By 2 days postinfection there is an apparent stimulation of growth in the mesenchyme or vascular bed, this growth being under and into the ectoderm itself. This growth is not apparent in routine histological preparations but is quite obvious in electron microscopic preparations (see Figs. 5 and 6). It was not seen in BSS-treated control CAM. In addition, many cell aggregates appear subectodermally in which the erythrocytes are centrally located. In some cases these aggregates do not appear to be open vessels but may represent blood island initiation, a normal potential of this mesoderm at all developmental stages. It is quite easy to distinguish the ectodermal cells from the underlying infiltrating cells because the latter are separated from the former by the adepidermal membrane and have distinct fine structural differences. The probability that initial infection involves the mesodermal derivatives is further strengthened by the finding of virus only within the mesenchyme or subectodermal cells at 48 hr. As the pocks form and increase in size, the ectodermal cells, erythrocytes, and fibroblasts seem to become intermixed. Even at 6 days, however, what appear to be ectodermal cells, on the basis of their electron opacity and desmosomal connections to adjacent cells, can be identified scattered throughout the pock. The presence of these cell attachments does not mean that the cells are all ectodermal, since desmosomes are present between the sinusoidal cells and ectodermal cells in the normal CAM (Fig. 2) and have been observed between vascular endothelial cells themselves (8) as well as fibroblasts (24).

At 4–6 days postinfection there is an apparent hyperplasia of the ectoderm. This late response of the ectoderm must be interpreted as resulting from altered physiological and/or physical relationships between the ectodermal cell population and the underlying mesenchymal or vascular cell population.

The response of the mesenchyme and vascular bed, and their proliferative potentials within the CAM are not out of line with respect to observations already made on this system. Embryologists have long used the CAM as a site of tissue explantation because of the high degree of vascularity which is established in the explant by proliferation of the CAM blood vessels. They were also the first

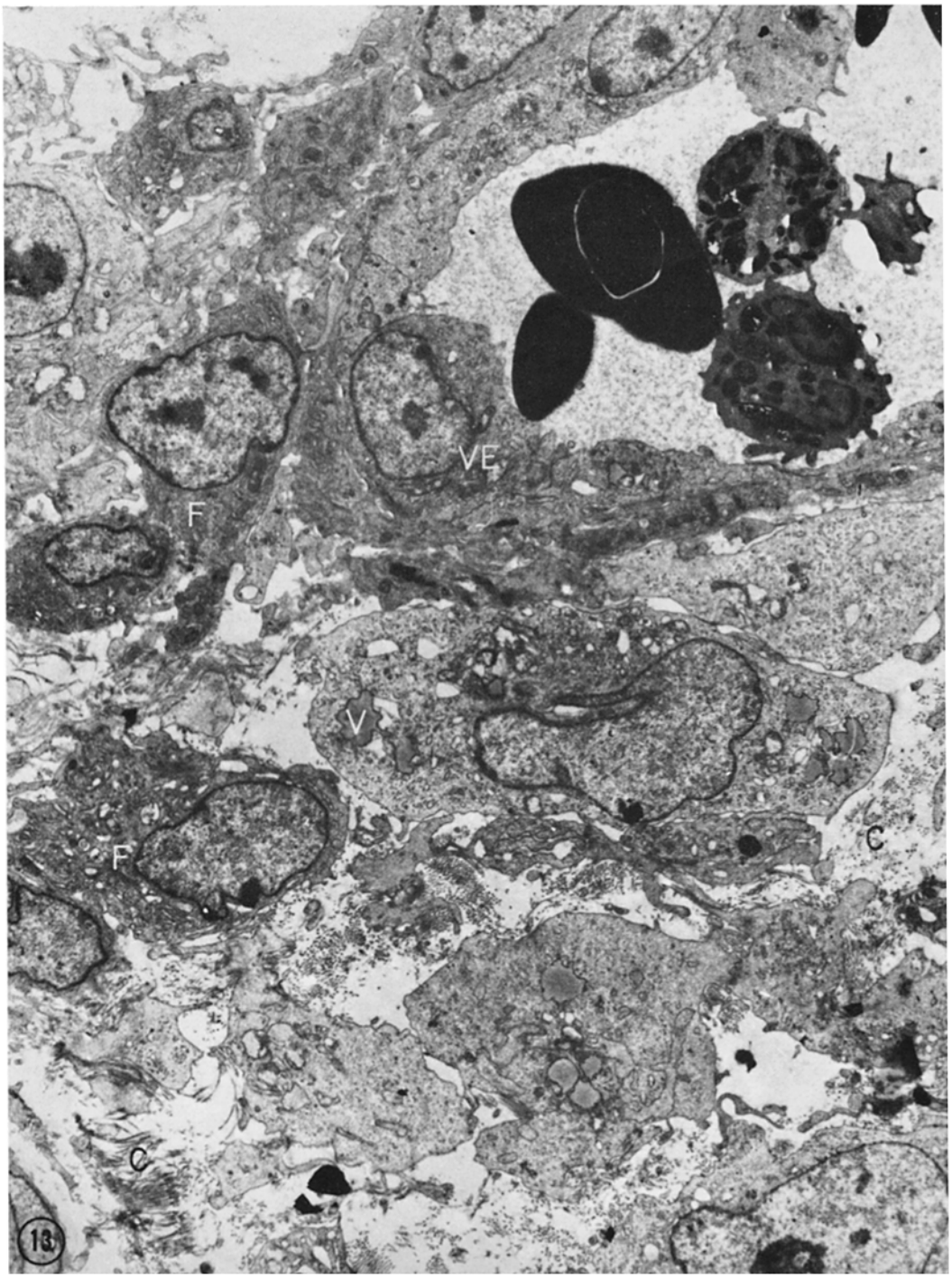


FIGURE 13 Electron micrograph of cells within a small discrete pock at 6 days postinfection. Three cell types are distinguishable: vascular endothelial cells (*VE*), fibroblasts (*F*), and vesiculated cells (*V*). Large amounts of intercellular collagen are also visible (*C*). OsO_4 . × 4600.

to show that a variety of agents (nonviral in nature) could cause ectodermal hyperplasia (10, 16, 17, 18, 22). A recent paper has shown that the developmental potential of the CAM ectoderm and mesoderm is indeed much greater than previously suspected (11).

The mesoderm of the CAM itself at the times (9 days of age) studied by most RSV investigators is indeed just terminating a very prolific state in its development. If the CAM is exposed to RSV at 6, 7, or 8 days of development it responds by producing diffuse lesions and occasional pocks. Only at 9 days or later are the characteristic pocks produced, the reproducibility of which can be used as an assay system (unpublished observations). It should be recalled that the formation of the allantoic sac is initiated at 3 days but that the sac does not contact the chorion, dorsal to the amnion, until 6–7 days, at which time there is an overt proliferation and extension of the mesenchymal vasculature. The subsequent penetration of mesenchymal vasculature through the ectoderm to establish the sinusoidal network occurs at 8–10 days. In the area where injections of RSV are made, these changes are completed at 9–10 days (23). Thus the greatest proliferative potential expressed at 6–7 days is within the vascular bed. If the virus indeed infects and transforms the cells of the vascular bed, one would expect the type of diffuse lesion which is observed at this time, the lesion showing mesodermal proliferation and little or no ectodermal hyperplasia. In addition, a great number of hemorrhagic lesions is usually seen in the CAM infected at 6 and 7 days of development (personal observation).

Extrahepatic hematopoiesis is known to occur within the extraembryonic membranes of the developing chick, and stimulation of this potential may also attend RSV infection and subsequent proliferation. Indeed the hemorrhagic lesions seen after injection of RSV into 1-day-old chicks may well result from infection and subsequent dilation or proliferation of capillary endothelium. Thus, continued hepatic erythropoiesis or even a true stimulation of extrahepatic erythropoiesis may account for the hemorrhagic lesions often reported to follow RSV infection (1, 4, 5, 9, 26, 28).

The probability that the RSV transforms mesodermal derivatives within this system is further strengthened by the observations on the transformation of other tissues. Ephrussi and Temin (7) have reported that RSV transforms iris epithelium in vitro. A close look at their paper reveals that the definitive conclusion that pigmented epithelium was transformed is not warranted. A great deal of vascular endothelium would also be anticipated in such a culture, and no evidence is presented to dispute the argument that such vascular cells may have given rise to the transformed population. In addition, it has been shown that pigment cells can transfer their pigment to other cells (2), and hence the existence of pigment within a cell does not preclude its production by that cell. Furthermore, kidney lesions, both hemorrhagic and sarcomatous, and hemorrhagic lesions of the spleen are frequent in newborn chicks injected with RSV (5). Two of these tissue aggregates (kidney and iris) are highly vascular (exceeded only by the lungs), and both are of mesodermal derivation. A recent publication on the transformation of RSV-infected chick limb buds grown on the CAM (4) reports that tumors appear within this system at the same time as the initiation of ossification, an event which is known to be related to vascular infiltration, and that at this time hemorrhagic lesions also appear. That RSV can cause the in vitro transformation of fibroblasts is a well-documented fact (27).

From the observations in this paper and other available information, it appears that the method by which RSV effects transformation in the CAM might not be as earlier proposed. We suggest that the following events take place when RSV is placed on the 9 day CAM. Upon dropping of the membrane by the creation of an artificial air space the sinusoidal spaces are ruptured, and at this time RSV has free access to the vascular cells, both sinusoidal and mesodermal. The RSV then acts by stimulating the proliferative potential of the vascular cells, either endothelial cells or pericytes, and it is this population of cells which then establishes the "tumor."

We believe that the ectodermal hyperplasia often seen by us is the result of a nonspecific stimulation by the tumor cell lysate or, perhaps, is even due to altered vascular or nutritive conditions deriving from the subepithelial growth of the tumor. This concept is presently being tested by isolating each of the tissue components of the CAM and exposing them to virus. The ultimate aim is to establish with certainty the precise cell population being transformed by exposure of the CAM to RSV.

This research was supported by grants from the National Research Council of Canada and the National Cancer Institute of Canada. Preliminary observations were reported at the American Association for Cancer Research, Philadelphia, 1965. Proceedings of the American Association for Cancer Research. **6**:63.

Received for publication 8 June 1967, and in revised form 9 October 1967.

REFERENCES

1. COATES, H. V. 1961. Hemangeomata in the CAM of the chick embryo following membrane inoculation of Rous sarcoma virus. *Federation Proc.* **20**:436.
2. CRUICKSHANK, C. N. D. 1965. Skin. *In* Cells and Tissues in Culture. Methods, biology, and physiology. E. N. Willmer, editor. Academic Press Inc., New York. **2**:549.
3. DOUGHERTY, R. M., P. J. SIMONS, and F. E. Chesterman. 1963. Biological properties of three variants of Rous sarcoma virus. *J. Natl. Cancer Inst.* **31**:1285.
4. DUNKEL, V. C., and V. GROUPÉ. 1965. Effects of Rous sarcoma virus on chicken embryo limb buds grafted onto the chorioallantoic membrane. *J. Natl. Cancer Inst.* **34**:204.
5. DURAN-REYNALS, F. 1940. A haemorrhagic disease occurring in chicks inoculated with the Rous and Fuginami viruses. *Yale J. Biol. Med.* **13**:77.
6. EBERT, JAMES D. 1959. The formation of muscle and muscle-like elements in the chorioallantoic membrane following inoculation of a mixture of cardiac microsomes and Rous sarcoma virus. *J. Exptl. Zool.* **142**:587.
7. EPHRUSSI, B., and H. M. TEMIN. 1960. Infection of chick iris epithelium with the Rous sarcoma virus in vitro. *Virology.* **11**:547.
8. FAWCETT, D. W. 1961. Intercellular bridges. *Exptl. Cell Res. Suppl.* **8**:174.
9. GROUPÉ, V., F. J. RAUSCHER, and W. R. BRYAN. 1957. Haemorrhagic disease and unusual hepatic lesions associated with intracerebral passage of Rous sarcoma virus in chicks. *J. Natl. Cancer Inst.* **19**:37.
10. HUXLEY, J. S., and P. D. F. MURRAY. 1924. A note on the reaction of the chick chorioallantois to grafting. *Anat. Record.* **28**:385.
11. KATO, Y., and Y. HAYASHI. 1963. The inductive transformation of the chorionic epithelium into skin derivatives. *Exptl. Cell Res.* **31**:599.
12. KEOGH, E. V. 1938. Ectodermal lesions produced by the virus of Rous sarcoma. *Brit. J. Exptl. Pathol.* **19**:1.
13. LEESON, T. S., and C. R. LEESON. 1963. The chorioallantois of the chick. Light and electron microscopic observations at various times of incubation. *J. Anat.* **97**:585.
14. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *Biochim. Biophys. Acta.* **9**: 409.
15. MILLONIG, G. 1961. Advantages of a phosphate buffer for OsO_4 solutions in fixation. *J. Appl. Phys.* **32**:1637.
16. MOSCONA, A. 1959. Squamous metaplasia and keratinization of chorionic epithelium of the chick embryo in egg and culture. *Develop. Biol.* **1**:1.
17. MOSCONA, A. 1960. Metaplastic changes in the chorioallantoic membrane. *Transplant. Bull.* **26**:120.
18. NICHOLAS, T. S., and D. RUDNICK. 1933. The development of embryonic rat tissues upon the chick chorioallantois. *J. Exptl. Zool.* **66**:193.
19. PALADE, G. E. 1961. Blood capillaries of the heart and other organs. *Circulation.* **24**:368.
20. PRINCE, A. M. 1958. Quantitative studies on Rous sarcoma virus. III. Virus multiplication and cellular response following infection of the chorioallantoic membrane of the chick embryo. *Virology.* **5**:435.
21. RICHARDSON, K. C., L. JARETT, and E. H. FINKE. 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.* **35**:313.
22. RICH, R. R., D. K. ROGERS, and F. E. DEADERS. 1965. Mesenchymal metaplasia of the chick CAM. A non-specific response to selected stimuli. *Exptl. Cell Res.* **40**:96.
23. ROMANOFF, A. L. 1960. The Avian Embryo. The Macmillan Company, New York.
24. ROSS, R., and J. K. GREENLEE, JR. 1966. Electron microscopy: Attachment sites between connective tissue cells. *Science.* **153**:997.
25. ROUS, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumour cells. *J. Exptl. Med.* **13**:397.
26. RUBIN, H. 1957. The production of virus by Rous sarcoma cells. *Ann. N.Y. Acad. Sci.* **68**:459.
27. RUBIN, H. 1959. A kinetic study of infection of chick embryo cells in vitro by RSV. *Virology.* **8**:209.
28. SVET-MOLDAVSKY, G. S. 1958. The pathogenicity of Rous sarcoma virus for mammals. Multiple cysts and haemorrhagic lesions of internal or-

gans in white rats after inoculation with Rous virus during the embryonic or newborn period. *Acta Virol.* **2:**1.

29. VIGIER, P. 1959. Reliability of titration of Rous sarcoma virus by the count of pocks produced on the egg chorioallantoic membrane. *Virology.* **8:** 41.
30. VRBA, M. 1965. Titration of Rous sarcoma virus on the chick embryo chorioallantoic membrane. *Neoplasma.* **12:**159.